

RESEARCH PAPER

Hydrogen water consumption prevents osteopenia in ovariectomized rats

Ji-Dong Guo*, Li Li*, Ya-Min Shi, Hua-Dong Wang and Shu-Xun Hou

Department of Orthopaedics, The First Affiliated Hospital of General Hospital of the People's Liberation Army, Beijing, China

Correspondence

Shu-Xun Hou, 51 Fucheng Road, Department of Orthopaedics, the First Affiliated Hospital of Chinese People's Liberation Army General Hospital, Beijing 100048, China. E-mail: houshuxun2012@hotmail.com

*The first two authors contributed equally to the work.

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BACKGROUND AND PURPOSE

Accumulating evidence indicates an important role of oxidative stress in the progression of osteoporosis. Recently, it was demonstrated that hydrogen gas, as a novel antioxidant, could selectively reduce hydroxyl radicals and peroxynitrite anion to exert potent therapeutic antioxidant activity. The aim of the present work was to investigate the effect of hydrogen water (HW) consumption on ovariectomy-induced osteoporosis.

EXPERIMENTAL APPROACH

Ovariectomized rats were fed with HW ($1.3 \pm 0.2 \text{ mg} \cdot \text{L}^{-1}$) for 3 months. Then, blood was collected and femur and vertebrae were removed for evaluation of the effect of HW on bone.

KEY RESULTS

HW consumption in ovariectomized rats had no significant effect on oestrogen production, but prevented the reduction of bone mass including bone mineral content and bone mineral density in femur and vertebrae, and preserved mechanical strength including ultimate load, stiffness, and energy, and bone structure including trabecular bone volume fraction, trabecular number, and trabecular thickness in femur, and preserved mechanical strength including ultimate load and stiffness, and bone structure including trabecular bone volume fraction and trabecular number in vertebrae. In addition, treatment with HW abated oxidative stress and suppressed IL-6 and TNF- α mRNA expressions in femur of ovariectomized rats; treatment with HW increased femur endothelial NOS activity and enhanced circulating NO level in ovariectomized rats.

CONCLUSIONS AND IMPLICATIONS

HW consumption prevents osteopenia in ovariectomized rats possibly through the ablation of oxidative stress induced by oestrogen withdrawal.

Abbreviations

BMC, bone mineral content; BMD, bone mineral density; BV/TV, bone volume/total volume; DPD, deoxypyridinoline; eNOS, endothelial NOS; HW, hydrogen water; MDA, malondialdehyde; NOx, nitrite/nitrate; Nox4, NADPH oxidase 4; ObS/BS, osteoblast surface to bone surface; OcS/BS, osteoclast surface to bone surface; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; SOD, superoxide dismutase; Tb.N, trabecular number; Tb.Th, trabecular thickness

Introduction

Osteoporosis is a disease characterized by the loss of bone mass and degeneration of bone microstructure. Patients suffering from this degenerative disease experience skeletal fra-

gility and consequently susceptibility to fracture, particularly of the wrist, spine and hip (Kanis *et al.*, 2009). Because the morbidity, mortality and economic cost associated with osteoporosis and subsequent fractures have increased in the last few years (Davison *et al.*, 2009), osteoporosis has been a

global problem and attracted more and more attention in public health.

Oxidative stress, resulting from excessive formation of reactive oxygen species (ROS) or dysfunction of antioxidant defence system, may play a role in postmenopausal bone loss (Baek *et al.*, 2010; Manolagas, 2010). Although supplement with antioxidants such as vitamin C or E had been proven protective against osteoporosis in rodents and *in vitro* studies (Nieves, 2005; Arslan *et al.*, 2011), the effect of these antioxidant vitamins in clinical trials was controversial (Wolf *et al.*, 2005; Talaulikar *et al.*, 2012). These agents are relatively inefficient and non-specific, which cannot target defined toxic ROS sources and deleterious redox-dependent signal pathways.

Recently, it has been proven that hydrogen gas, a highly flammable gas, has amazing antioxidant property (Ohsawa *et al.*, 2007). Compared with most commonly used antioxidants such as vitamin C and vitamin E, molecular hydrogen has two unique characteristics. First, molecular hydrogen selectively reduces cytotoxic ROS such as the hydroxyl radical and does not react with other ROS that possesses physiological roles. Second, because the molecular hydrogen is small and electrically neutral, it easily penetrates membranes and enters cells and organelles including nucleus and mitochondria, where normal antioxidant cannot arrive (Ohsawa *et al.*, 2007).

In the present work, we explored whether chronic hydrogen water (HW) consumption prevented osteopenia in ovariectomized rats.

Methods

Animals

All the animals were fed under controlled temperature (21–23°C), 12 h light and 12 h dark cycles (light, 08:00–20:00 h; darkness, 20:00–08:00 h), and free access to food and tap water. All the animals used in this work received humane care in compliance with institutional animal care guidelines. All the surgical and experimental procedures were in accordance with institutional animal care guidelines, and were approved by the Local Institutional Committee. Sprague–Dawley rats (2 months old, 200–250 g) were purchased from the Vital-Aiver Animal Ltd. (Beijing, China). Chemicals, drugs and reagents were obtained from Sigma Chemical (St. Louis, MO, USA) unless otherwise stated. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Study design

Animals were divided into four groups of 60 animals each and treated for 3 months as follows: (i) sham-operated rats drinking with normal water; (ii) sham-operated rats drinking with HW; (iii) ovariectomized rats drinking with normal water; and (iv) ovariectomized rats drinking with HW. Three months later, the body weight and food intake of rats were determined. Plasma pH, oestrogen, osteocalcin, malondialdehyde (MDA) and nitrite/nitrate (NOx) content were determined. The mRNA levels of superoxide dismutase 1 (SOD1), SOD3, catalase, NADPH oxidase 4 (Nox4), p22phox, IL-6,

TNF- α and endothelial NOS (eNOS) in femur were determined using quantitative real-time PCR (qRT-PCR) method. The bone mass, mechanical properties, and trabecular bone morphometry of femur and lumbar vertebra were measured.

HW

Hydrogen was dissolved in water for 4 h under the pressure of 0.4 MPa as the method described by Ohsawa *et al.* (2007). The saturated HW (1.3 ± 0.2 mg·L⁻¹) was stored under atmospheric pressure at 4°C in an aluminium bag with no dead volume for use within 1 week. HW was sterilized by gamma radiation and freshly prepared every week. Each day, HW from the aluminium bag was placed in a closed glass vessel, which ensured that the hydrogen concentration was greater than 0.8 mg·L⁻¹ after 1 day.

Ovariectomy

Ovariectomy was performed under isoflurane anaesthesia. A single dorsal incision was made in the skin and through it two lateral incisions in the muscle layer were made, approximately half a centimetre below each kidney. The ovaries were extruded through the incision, ligated off and removed. Treatment was started after a 1 week recovery period.

Measurement of plasma oestrogen, osteocalcin, MDA, DPD and NOx

The concentration of plasma oestrogen was measured using a commercially available immunosorbent assay kit (Cayman, Ann Arbor, MI, USA). The concentration of serum osteocalcin was measured using a radioimmunoassay kit (China Institute of Atomic Energy, Beijing, China). Plasma was used for the determination of MDA using a kit (Cayman). Urinary deoxy-pyridinoline (DPD) excretion was measured using an OSTEOLINKS-DPD EIA kit (Sumitomo Seiyaku, Osaka, Japan), and the data were corrected for the urinary creatinine concentration. Creatinine was quantified with QuantiChrom Creatinine Assay Kits (BioAssay Systems, Hayward, CA, USA). The levels of NOx, the stable end products of NO, in plasma were measured using a total nitrite/nitrate assay kit (Dojindo, Kumamoto, Japan) that employs the Griess method (Green *et al.*, 1982).

Measurement of cytokines in serum

Immediately after decapitation, a 2 mL blood sample was collected and serum sample was obtained by centrifugation at 1290× g for 15 min and stored at –20°C for assay within 2 months. Serum TNF- α and IL-6 levels were evaluated using ELISA kits (R&D Systems, Minneapolis, MN, USA).

BMC, BMD and bone area measurement

Bone mineral content (BMC), bone mineral density (BMD), and cross-section bone area measurement of femur and vertebrae were performed as previously described (Lei *et al.*, 2009). BMD and BMC were measured *ex vivo* with a dual energy X-ray absorptiometry NORLAND XR-46 (Norland Co., Fort Atkinson, WI, USA) using the small-animal program set to a high-resolution mode. Samples were placed on an acrylic platform of uniform 38.1 mm thickness. Regional high-resolution scans of both femurs and the lumbar spine (L4) were performed using a 1.524-mm-diameter collimator with

0.30516 mm point resolution and 0.64516 mm line spacing. Selection of region of interests was performed as previously described (Akiyama *et al.*, 1999; Shiraishi *et al.*, 2009). The coefficient of variation was 3.0% for BMC and 1% for BMD.

Measurement of mechanical properties

Using a mechanical strength analyser (TK-252CC; Muromachi Kikai Co., Ltd, Tokyo, Japan), the mechanical strength of the lumbar vertebra (L5) and left femur was measured as the method described previously (Mosekilde *et al.*, 1993; Katsumata *et al.*, 1995).

For the compression test, the planoparallel surfaces were obtained by removing the cranial and caudal ends of the vertebral specimen. From the vertebral body (L5), a central cylinder with planoparallel ends and a height of approximately 5 mm was obtained. A compression force was applied to the specimen in the craniocaudal direction using a steel disk at a deformation rate of 2.5 mm·min⁻¹. The ultimate compressive load (N), the stiffness (N·mm⁻¹) and the energy (mJ) were calculated as the mechanical properties directly from the load-displacement curve. For the three-point bending test, the left femur was placed on a special holding device with supports located 12 mm apart. A bending force was applied with the cross head at a speed of 20 mm min⁻¹, until a fracture occurred. From the load-displacement curve, the ultimate compressive load (N), the stiffness (N·mm⁻¹) and the energy (mJ) were obtained.

qRT-PCR analysis

The femur was excised and immediately frozen in liquid nitrogen until required. The frozen distal femur was put in a RNase-free mortar and pestle that contained liquid nitrogen and ground to a fine powder immersed in liquid nitrogen. The frozen powder was transferred into a tube containing Trizol (Life Technologies Inc., Gaithersburg, MD, USA) and total RNA was isolated, according to the manufacturer's protocol. RT-PCR analysis was performed with a QuantiTect™ SYBR® Green PCR (Tiangen, Shanghai, China) according to the manufacturer's instructions. The RT-PCR data were based on SYBR green amplification. The sequences of primers are listed in Supporting Information Table S1 (shown in supporting data). The highly specific measurement of mRNA was carried out for SOD1, SOD3, catalase, Nox4, p22phox, TNF- α , IL-6, eNOS, Runx2 and GAPDH using the LightCycler system (Bio-Rad, Carlsbad, CA, USA). Each sample was run and analysed in duplicate. Target mRNA levels were adjusted as the values relative to GAPDH, which was used as the endogenous control to ensure equal starting amounts of cDNA. The fold change relative to control values was obtained and used to express the experimental change in gene expression.

NF- κ B activity

To analyse the specific binding activity of nuclear p65 NF- κ B proteins, transcription factor ELISA was performed using nuclear extracts following the manufacturer's instruction (NF- κ B TransAM kit; Active Motif, Carlsbad, CA, USA) and procedures described previously (Cho *et al.*, 2002).

Western blotting analysis

The protein concentration was determined with BSA as a standard by a Bradford assay. Equal amount of protein

preparations (10 μ g in 10 μ L buffer) were run on SDS-polyacrylamide gels, electrotransferred to polyvinylidene difluoride membranes, and blotted with a primary antibody against I κ B α (Santa Cruz Biotechnology, Inc., CA, USA) overnight at 4°C using slow rocking. Then, they were blotted with HRP-conjugated secondary antibody (1:5000) and HRP-conjugated monoclonal antibody against GAPDH (1:10 000). Immunoreactive bands were detected by a chemiluminescent reaction (ECL kit; Amersham Pharmacia, San Francisco, CA, USA).

Plasma pH measurement

Plasma pH was determined by using blood-gas analysis in an automatic blood-gas analyser (ABL system; Radiometer, Copenhagen, Denmark).

Micro-CT

Trabecular bone morphometry within the metaphyseal region of distal femur and L5 vertebral body was quantified using micro-CT (μ CT40, Scanco Medical AG, 10.5 μ m voxel size, 55 kVp, 145 μ A) with a threshold value of 240. The L5 vertebral body and distal femur were scanned in 250 slices (thickness, 13 μ m) in the dorsoventral direction. Three-dimensional reconstruction of bone was performed using the triangulation algorithm. Volumetric regions for trabecular analysis were selected within the endosteal borders to include the central 80% of vertebral height and secondary spongiosa of femoral metaphyses located ~6% of the total length from the growth plate. Trabecular morphometry was characterized by measuring the bone volume fraction (bone volume/total volume, BV/TV), trabecular thickness (Tb.Th) and trabecular number (Tb.N).

Bone histomorphometry

The lumbar vertebrae (L5) and distal femur were incubated with the Villanueva bone stain for 7 days, dehydrated in graded ethanols and xylene, and embedded undecalcified in methyl methacrylate. Frontal sections (4 μ m thick) were cut with vertical bed microtomes (Leica, Rockleigh, NJ, USA) and affixed to slides pre-coated with a 1% gelatin solution. Then they were stained according to the Von Kossa method with a tetrachrome counterstain (Polysciences, Warrington, PA, USA). Regions for trabecular analysis were selected within the endosteal borders to include the central 80% of vertebral height and secondary spongiosa of femoral metaphyses located ~6% of the total length from the growth plate. Histomorphometric data were collected with the Bioquant Bone Morphometry System (R&M Biometrics Corp., Nashville, TN, USA). Osteoclast and osteoblast surfaces were measured in the 4 μ m stained sections and expressed as percentages of total cancellous bone surface.

Statistical analysis

All the data are presented as mean \pm standard deviations. Comparison among groups was analysed using a two-way analysis of variance followed by Bonferroni *t*-test. *P* < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS 11.0.0 software (SPSS Inc., Chicago, IL, USA).

Results

Effects of HW on bone of ovariectomized rats

Ovariectomy in rats resulted in increased body weight, decreased plasma oestrogen content, increased plasma osteocalcin content and urinary DPD, but had no significant effect on food intake. HW consumption in ovariectomized rats had no significant effect on body weight and plasma oestrogen and pH levels, but it significantly decreased serum osteocalcin levels and urinary DPD content (Table 1). HW consumption in sham-operated rats had no significant effect on body weight, plasma oestrogen content, serum osteocalcin content, food intake and plasma pH levels.

Ovariectomy in rats significantly decreased femoral bone mass including BMC and BMD, and lowered femoral

mechanical strength including ultimate load, the stiffness, and the energy, and disrupted femoral bone structure marked by decreased BV/TV, Tb.N and Tb.Th, but had no effect on femoral bone area (Table 2). HW consumption in ovariectomized rats increased femoral BMC, BMD, ultimate load, the stiffness, the energy, BV/TV, Tb.N and Tb.Th.

Ovariectomy in rats significantly decreased vertebral bone mass including BMC and BMD, and lowered vertebral mechanical strength including ultimate load and the stiffness, and disrupted vertebral bone structure marked by decreased BV/TV and Tb.N, but had no effect on vertebral bone area or mechanical energy or Tb.Th (Table 3). HW consumption in ovariectomized rats increased vertebral BMC, BMD, ultimate load, the stiffness, BV/TV and Tb.N.

Table 1

Effects of HW on ovariectomized rats

	Sham	Sham+ HW	OVX	OVX+ HW
BW (g)	352 ± 38	350 ± 40	409 ± 29 ^a	413 ± 37 ^a
Food intake (g·day ⁻¹)	20.3 ± 2.8	19.5 ± 3.5	21.1 ± 3.0	20.6 ± 4.1
Plasma oestrogen (pg·mL ⁻¹)	23.57 ± 5.32	25.18 ± 4.49	5.99 ± 0.97 ^a	6.43 ± 1.13 ^a
Plasma pH	7.53 ± 0.13	7.55 ± 0.14	7.52 ± 0.15	7.51 ± 0.16
Serum osteocalcin (ng·mL ⁻¹)	0.118 ± 0.023	0.120 ± 0.030	0.203 ± 0.037 ^a	0.149 ± 0.038 ^{a,b}
DPD (nM·nM per creatinine)	21.8 ± 3.3	20.7 ± 4.1	45.9 ± 6.2 ^a	27.5 ± 5.2 ^{a,b}

Values are represented as mean ± SD.

^a*P* < 0.05 versus the sham-operated group.

^b*P* < 0.05 versus the ovariectomized group.

Sham, sham-operated group; OVX, ovariectomized group; BW, body weight; *n* = 10–12 in each group.

Table 2

Effects of HW on the femur of ovariectomized rats

	Sham	Sham+ HW	OVX	OVX+ HW
BMD (g·cm ⁻²)	0.185 ± 0.021	0.190 ± 0.017	0.155 ± 0.018 ^a	0.176 ± 0.0191 ^b
BMC (g)	0.276 ± 0.035	0.280 ± 0.021	0.235 ± 0.027 ^a	0.269 ± 0.028 ^b
Area (mm ²)	6.7 ± 0.6	7.0 ± 0.5	7.1 ± 0.5	6.9 ± 0.6
Ultimate load (N)	217 ± 49	221 ± 55	149 ± 42 ^a	198 ± 46 ^b
Stiffness (N·mm ⁻¹)	477 ± 93	483 ± 91	325 ± 72 ^a	440 ± 101 ^b
Energy (mJ)	78 ± 19	80 ± 16	54 ± 12 ^a	70 ± 14 ^b
BV/TV (%)	33.1 ± 5.0	33.5 ± 4.3	23.4 ± 3.9 ^a	29.3 ± 4.2 ^b
Tb.N	5.6 ± 1.3	5.8 ± 1.4	4.1 ± 0.6 ^a	4.8 ± 0.8 ^b
Tb.Th (μm)	119.3 ± 8.3	115.4 ± 9.2	104.7 ± 8.4 ^a	112.1 ± 8.7 ^b
ObS/BS (%)	5.56 ± 0.98	5.28 ± 1.16	7.22 ± 3.25 ^a	6.02 ± 1.39 ^b
OcS/BS (%)	1.34 ± 0.55	1.29 ± 0.63	4.39 ± 1.26 ^a	2.38 ± 0.81 ^b

BMD, BMC and bone area were measured with a dual energy X-ray absorptiometry NORLAND XR-46. The ultimate compressive load, the stiffness and the energy were measured using a mechanical strength analyser. BV/TV, Tb.N and Tb.Th were measured by uCT. ObS/BS and OcS/BS were measured by histomorphometry. Values are represented as mean ± SD.

^a*P* < 0.05 versus the sham-operated group.

^b*P* < 0.05 versus the ovariectomized group.

Sham, sham-operated group; OVX, ovariectomized group; *n* = 10–12 in each group.

Table 3

Effects of HW on the lumbar vertebra of ovariectomized rats

	Sham	Sham+ HW	OVX	OVX+ HW
BMD (g·cm ⁻²)	0.206 ± 0.024	0.215 ± 0.030	0.174 ± 0.019 ^a	0.195 ± 0.025 ^b
BMC (g)	0.386 ± 0.040	0.392 ± 0.034	0.318 ± 0.037 ^a	0.366 ± 0.040 ^b
Area (mm ²)	31.0 ± 3.0	31.7 ± 2.9	31.6 ± 4.1	32.0 ± 3.6
Ultimate load (N)	376 ± 57	383 ± 60	302 ± 50 ^a	357 ± 55 ^b
Stiffness (N·mm ⁻¹)	1127 ± 121	1085 ± 109	853 ± 100 ^a	995 ± 93 ^b
Energy (mJ)	138 ± 33	132 ± 35	126 ± 44	130 ± 42
BV/TV (%)	43.4 ± 5.1	42.5 ± 4.2	32.6 ± 3.9 ^a	39.7 ± 4.1 ^b
Tb.N	5.3 ± 0.7	5.2 ± 1.1	4.0 ± 0.8 ^a	4.8 ± 0.9 ^b
Tb.Th (μm)	80.7 ± 6.8	81.3 ± 9.0	76.1 ± 7.4	77.7 ± 6.5
ObS/BS (%)	3.45 ± 0.55	3.77 ± 0.71	7.80 ± 1.21 ^a	4.64 ± 1.06 ^{a,b}
OcS/BS (%)	0.55 ± 0.12	0.53 ± 0.15	1.28 ± 0.42 ^a	0.90 ± 0.36 ^b

BMD, BMC and bone area were measured with a dual energy X-ray absorptiometry NORLAND XR-46. The ultimate compressive load, the stiffness and the energy were measured using a mechanical strength analyser. BV/TV, Tb.N and Tb.Th were measured by uCT. ObS/BS and OcS/BS were measured by histomorphometry. Values are represented as mean ± SD.

^a*P* < 0.05 versus the sham-operated group.

^b*P* < 0.05 versus the ovariectomized group.

Sham, sham-operated group; OVX, ovariectomized group; *n* = 10–12 in each group.

When compared with sham-operated rats, increased osteoblast surface to bone surface (ObS/BS) and osteoclast surface to bone surface (OcS/BS) were found in femur and vertebra of ovariectomized rats. HW consumption in ovariectomized rats decreased OcS/BS and ObS/BS in both femur and vertebra significantly.

HW consumption in sham-operated rats had no significant effect on BMC, BMD, bone area, ultimate load, the stiffness, the energy, BV/TV, Tb.N, Tb.Th, ObS/BS, and OcS/BS in femur and vertebrae.

Effects of HW on oxidative stress in ovariectomized rats

Ovariectomy in rats resulted in oxidative stress, marked by increased MDA content in plasma (Figure 1A), reduced expression of antioxidative defence enzymes including SOD1 (Figure 1B), SOD3 (Figure 1C), and catalase (Figure 1D), and enhanced expression of oxidative enzymes including Nox4 (Figure 1E) and p22phox (Figure 1F) in femur. HW consumption in ovariectomized rats reduced MDA level and enhanced SOD1, SOD3, and catalase mRNA expression, and inhibited Nox4 expression, but had no significant effect on p22phox expression in femur. HW consumption in sham-operated rats had no significant effect on plasma MDA content and SOD1, SOD3, catalase, Nox4, p22phox mRNA levels in femur.

Effects of HW on cytokines in ovariectomized rats

Ovariectomy in rats resulted in increased TNF-α level in serum (Figure 2A), but did not affect IL-6 production (Figure 2B). In femur, ovariectomy enhanced mRNA expression of TNF-α (Figure 2C) and IL-6 (Figure 2D) and activity of NF-κB p65 (Figure 2E), and decreased IκBα (Figure 2F) protein

expression. HW consumption in ovariectomized rats decreased TNF-α level in serum (Figure 2A) and reduced TNF-α (Figure 2C) and IL-6 (Figure 2D) mRNA expression and suppressed NF-κB p65 (Figure 2E) activity and enhanced IκBα (Figure 2F) protein expression in femur. HW consumption in sham-operated rats had no significant effect on TNF-α and IL-6 levels in serum and TNF-α and IL-6 mRNA expression, NF-κB p65 activity and IκBα protein expression in femur.

Effects of HW on NO formation in ovariectomized rats

In ovariectomized rats, plasma NOx content was lower than that in sham-operated rats (Figure 3A). RT-PCR results showed that eNOS mRNA expression was also lower in femur of ovariectomized rats (Figure 3B). HW consumption in ovariectomized rats enhanced plasma NOx content and up-regulated femoral eNOS mRNA expression. HW consumption in sham-operated rats had no significant effect on plasma NOx content and femoral eNOS mRNA expression.

Discussion

Recently, Sun *et al.* demonstrated that treatment with molecular hydrogen alleviates microgravity-induced bone loss *in vivo* and *in vitro* (Sun *et al.*, 2012) and first revealed the protective effect of molecular hydrogen on bone. In this study, although chronic treatment with HW had no significant effect on circulating oestrogen, it effectively alleviated osteopenia in ovariectomized rats. Therefore, the beneficial effect of HW on osteoporosis induced by oestrogen deficiency was independent of oestrogen. Treatment with hydrogen

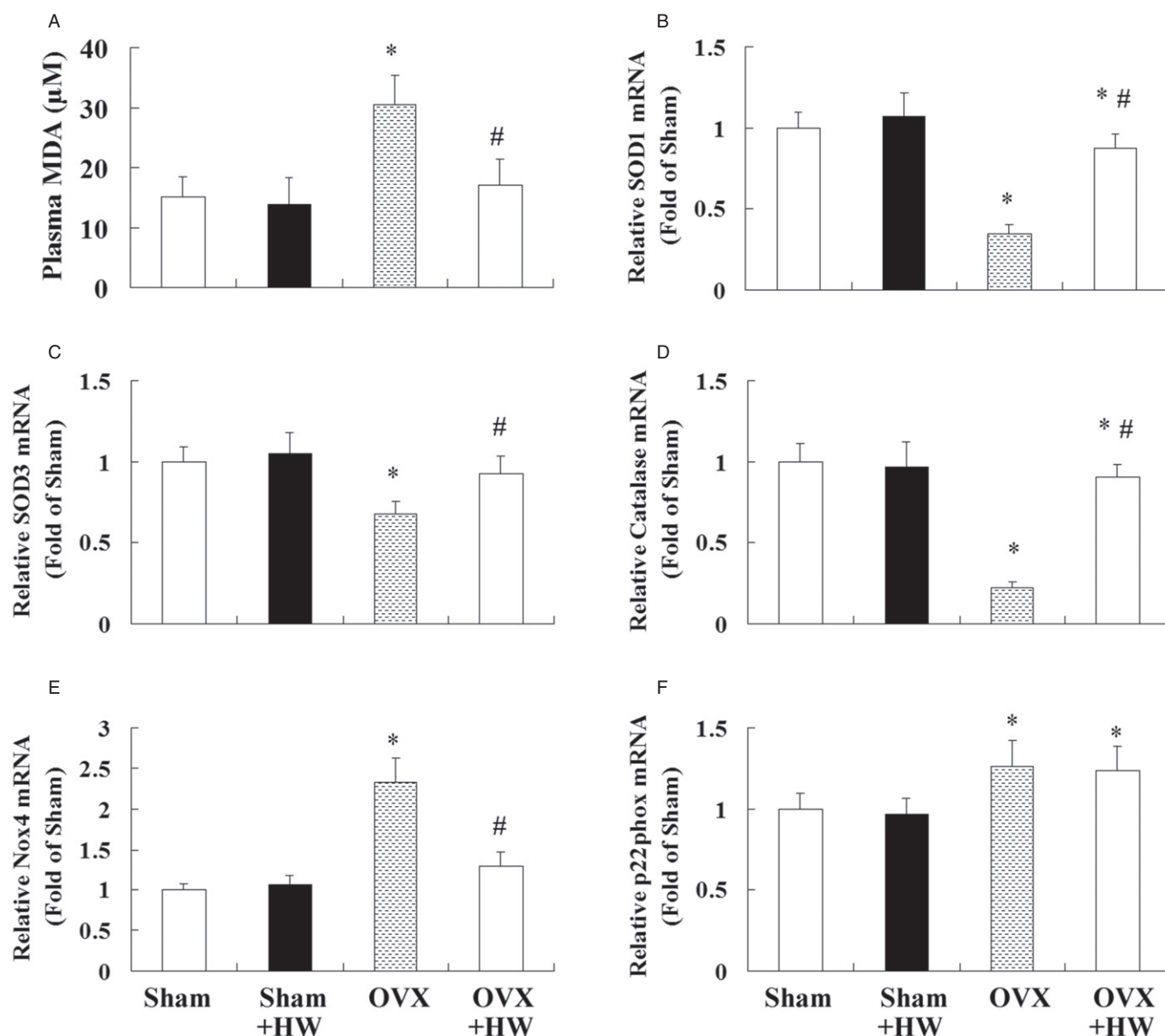


Figure 1

Effect of HW on oxidative stress in ovariectomized rats. Plasma MDA (A) content was measured using an ELISA kit. Femur SOD1 (B), SOD3 (C), catalase (D), Nox4 (E) and p22phox (F) mRNA levels were determined using qRT-PCR method. Sham, sham-operated group; OVX, ovariectomized group; $n = 10$ – 12 in each group; * $P < 0.05$ versus the sham-operated group; # $P < 0.05$ versus the ovariectomized group.

molecule prevents osteopenia in ovariectomized rats, possibly through the ablation of the oxidative stress induced by oestrogen withdrawal.

In this study, treatment with HW suppressed the increased osteoclast surface in ovariectomized rats. The cytokine IL-6 has been proposed to act as a stimulator of osteoclast formation and activity, in particular following loss of oestrogen (Jilka *et al.*, 1992). IL-6-deficient mice are protected from bone loss caused by oestrogen depletion (Poli *et al.*, 1994). The femoral IL-6 mRNA expression was found suppressed by treatment with HW. Therefore, treatment with HW might suppress osteoclastogenesis through inhibition of IL-6 expression in ovariectomized rats.

In the current study, treatment with HW abated oxidative stress, which might result from enhanced expression of antioxidant enzymes including SOD1, SOD3, and catalase, and decreased Nox4 expression.

SOD and catalase, as two important defences against excessive ROS in bone tissue, are responsible to reduce the superoxide radicals to water (Noor *et al.*, 2002; Muller *et al.*, 2006). Mice deficient in SOD1 exhibited lower BMD and reduced bone strength (Smietana *et al.*, 2010). Decreased SOD activity was found accompanied with increased oxidative stress in aged rodents and postmenopausal women (Ozgocmen *et al.*, 2007; Zhang *et al.*, 2011). In addition, catalase administration was shown to prevent oophorectomy-

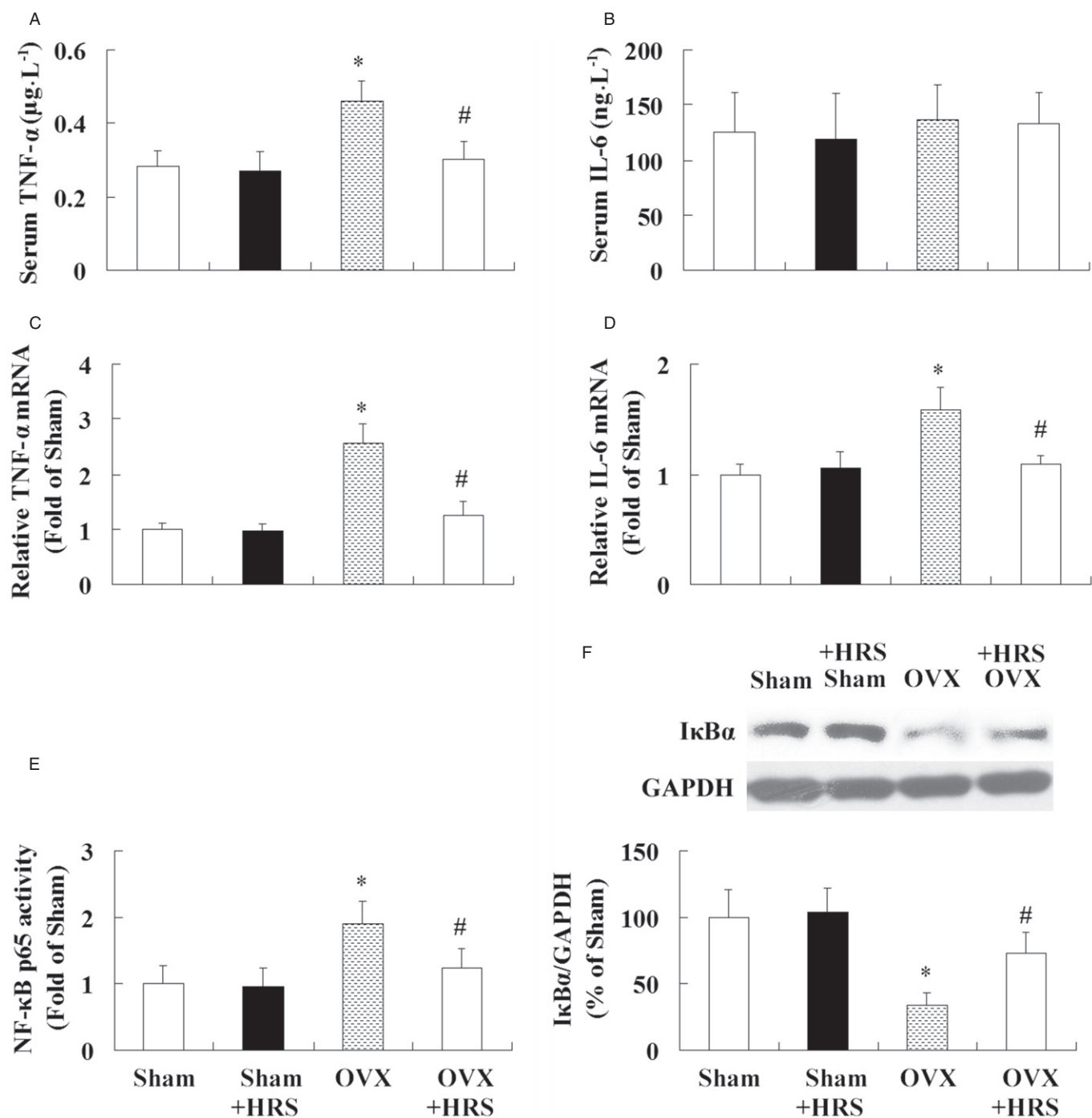


Figure 2

Effect of HW on cytokines in ovariectomized rats. Serum TNF- α (A) and IL-6 (B) concentrations were measured using ELISA kits. Femur TNF- α (C) and IL-6 (D) mRNA levels were determined using qRT-PCR method. NF- κB activity (E) was determined with ELISA kits. I $\kappa\text{B}\alpha$ protein expression (F) was determined through Western blotting analysis. Sham, sham-operated group; OVX, ovariectomized group; $n = 10\text{--}12$ in each group; * $P < 0.05$ versus the sham-operated group; # $P < 0.05$ versus the ovariectomized group.

induced bone loss (Lean *et al.*, 2005). Those results suggest that SOD and catalase activities may be involved in the development of oxidative stress-related osteoporosis. In the current study, treatment with HW increased the SOD and catalase activities in ovariectomized rats. Expressions of

Nox4 and p22phox, as the major sources of intracellular ROS in bone (Mandal *et al.*, 2010), were found enhanced in ovariectomized rats. Treatment with HW significantly inhibited Nox4 expression but did not affect p22phox expression in femur.

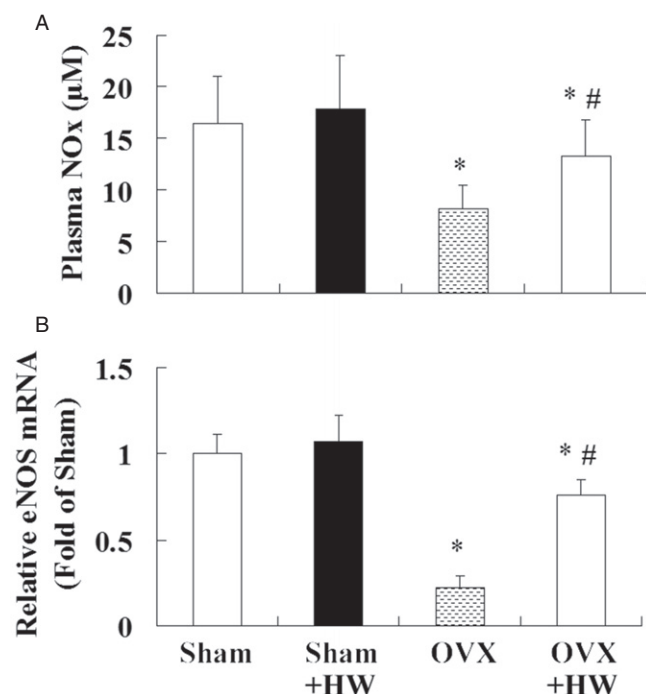


Figure 3

Effect of HW on NO formation in ovariectomized rats. Plasma NOx (A) content was measured with the Griess method. Femur eNOS mRNA level (B) was measured using qRT-PCR method. $n = 10$ – 12 in each group; * $P < 0.05$ versus the sham-operated group; # $P < 0.05$ versus the ovariectomized group.

Circulating NO levels and femur eNOS expression were found reduced in ovariectomized rats. NO derived from eNOS in bone tissue is one of the key local mediators and crucial second messengers of systemic hormones including calcitonin gene-related peptide, parathyroid hormone and sex steroids, particularly of oestrogen (Wimalawansa *et al.*, 1996; Hukkanen *et al.*, 2003). Therefore, enhanced NO level might contribute to the beneficial effect of HW on bone in ovariectomized rats. In addition, as eNOS was only one of three NO synthases in bone, relationship between enhanced NO level and up-regulated eNOS expression after HW consumption remained unclear and required further investigation. Here, our results only revealed that HW consumption enhanced NO level, which might be contributed to up-regulated eNOS expression, at least in part.

In this study, treatment with HW significantly decreased plasma TNF- α level and femoral TNF- α expression in ovariectomized rats. TNF- α has been shown to decrease osteoblastic bone formation through the suppression of osteoblast proliferation, induction of osteoblast apoptosis and inhibition of osteoblast differentiation (Gilbert *et al.*, 2000). In addition, TNF- α induced bone resorption by stimulating the production of osteoclast-like multinucleated cells and by increasing the bone-resorbing activity of formed osteoclasts (Kobayashi *et al.*, 2000). The suppression of molecular hydrogen on TNF- α and IL-6 might be secondary to its anti-oxidative effect. Excess ROS activates the redox-sensitive transcription factor NF- κ B, and leads to augmentation of its expression and

activity (Almeida *et al.*, 2010). Increased expression and activity of NF- κ B induce gene transcription of cytokines including IL-6 and TNF- α to increase their production (Janssen-Heininger *et al.*, 2000; Sanlioglu *et al.*, 2001). NF- κ B activation in ovariectomized rats was found inhibited by treatment with HW.

Conclusion

HW consumption prevents osteopenia in ovariectomized rats, possibly through the ablation of oxidative stress induced by oestrogen withdrawal.

Conflict of interest

None to declare.

References

- Akiyama Y, Hara K, Kobayashi M, Tomiuga T, Nakamura T (1999). Inhibitory effect of vitamin K2 (menatetrenone) on bone resorption in ovariectomized rats: a histomorphometric and dual energy X-ray absorptiometric study. *Jpn J Pharmacol* 80: 67–74.
- Almeida M, Han L, Ambrogini E, Bartell SM, Manolagas SC (2010). Oxidative stress stimulates apoptosis and activates NF-kappaB in osteoblastic cells via a PKCbeta/p66shc signaling cascade: counter regulation by estrogens or androgens. *Mol Endocrinol* 24: 2030–2037.
- Arslan A, Orkun S, Aydin G, Keles I, Tosun A, Arslan M *et al.* (2011). Effects of ovariectomy and ascorbic acid supplement on oxidative stress parameters and bone mineral density in rats. *Libyan J Med* 6: 1–9.
- Baek KH, Oh KW, Lee WY, Lee SS, Kim MK, Kwon HS *et al.* (2010). Association of oxidative stress with postmenopausal osteoporosis and the effects of hydrogen peroxide on osteoclast formation in human bone marrow cell cultures. *Calcif Tissue Int* 87: 226–235.
- Cho HY, Jedlicka AE, Reddy SP, Kensler TW, Yamamoto M, Zhang LY *et al.* (2002). Role of Nrf2 in protection against hyperoxic lung injury in mice. *Am J Respir Cell Mol Biol* 26: 175–182.
- Davison KS, Kendler DL, Ammann P, Bauer DC, Dempster DW, Dian L *et al.* (2009). Assessing fracture risk and effects of osteoporosis drugs: bone mineral density and beyond. *Am J Med* 122: 992–997.
- Gilbert L, He X, Farmer P, Boden S, Kozlowski M, Rubin J *et al.* (2000). Inhibition of osteoblast differentiation by tumor necrosis factor-alpha. *Endocrinology* 141: 3956–3964.
- Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982). Analysis of nitrate, nitrite, and [15 N] nitrate in biological fluids. *Anal Biochem* 126: 131–138.
- Hukkanen M, Platts LA, Lawes T, Girgis SI, Konttinen YT, Goodship AE *et al.* (2003). Effect of nitric oxide donor nitroglycerin on bone mineral density in a rat model of estrogen deficiency-induced osteopenia. *Bone* 32: 142–149.

- Janssen-Heininger YMW, Poynter ME, Baeuerle PA (2000). Recent advances towards understanding redox mechanisms in the activation of nuclear factor κ B. *Free Radic Biol Med* 28: 1317–1327.
- Jilka RL, Hangoc G, Girasole G, Passeri G, Williams DC, Abrams JS *et al.* (1992). Increased osteoclast development after estrogen loss: mediation by interleukin-6. *Science* 257: 88–91.
- Kanis JA, Johansson H, Oden A, McCloskey EV (2009). Assessment of fracture risk. *Eur J Radiol* 71: 392–397.
- Katsumata T, Nakamura T, Ohnishi H, Sakurawa T (1995). Intermittent cyclical etidronate treatment maintains the mass, structure and the mechanical property of bone in ovariectomized rats. *J Bone Miner Res* 10: 921–931.
- Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). NC3Rs Reporting Guidelines Working Group. *Br J Pharmacol* 160: 1577–1579.
- Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S *et al.* (2000). Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. *J Exp Med* 191: 275–286.
- Lean JM, Jagger CJ, Kirstein B, Fuller K, Chambers TJ (2005). Hydrogen peroxide is essential for estrogen-deficiency bone loss and osteoclast formation. *Endocrinology* 146: 728–735.
- Lei Z, Xiaoying Z, Xingguo L (2009). Ovariectomy-associated changes in bone mineral density and bone marrow haematopoiesis in rats. *Int J Exp Pathol* 90: 512–519.
- Mandal CC, Ganapathy S, Gorin Y, Mahadev K, Block K, Abboud HE *et al.* (2010). Reactive oxygen species derived from Nox4 mediate BMP2 gene transcription and osteoblast differentiation. *Biochem J* 433: 393–402.
- Manolagas SC (2010). From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis. *Endocr Rev* 31: 266–300.
- McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. *Br J Pharmacol* 160: 1573–1576.
- Mosekilde L, Danielsen CC, Knudsen UB (1993). The effects of aging and ovariectomy on the vertebral bone mass and biomechanical properties of mature rats. *Bone* 14: 1–6.
- Muller FL, Song W, Liu Y, Chaudhuri A, Piek-Dahl S, Strong R *et al.* (2006). Absence of CuZn superoxide dismutase leads to elevated oxidative stress and acceleration of age-dependent skeletal muscle atrophy. *Free Radic Biol Med* 40: 1993–2004.
- Nieves JW (2005). Osteoporosis: the role of micronutrients. *Am J Clin Nutr* 81: 1232S–1239S.
- Noor R, Mittal S, Iqbal J (2002). Superoxide dismutase-applications and relevance to human diseases. *Med Sci Monit* 8: 210–215.
- Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K *et al.* (2007). Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nat Med* 13: 688–694.
- Ozgocmen S, Kaya H, Fadillioglu E, Aydogan R, Yilmaz Z (2007). Role of antioxidant systems, lipid peroxidation, and nitric oxide in postmenopausal osteoporosis. *Mol Cell Biochem* 295: 45–52.
- Poli V, Balena R, Fattori E, Markatos A, Yamamoto M, Tanaka H *et al.* (1994). Interleukin-6 deficient mice are protected from bone loss caused by estrogen depletion. *EMBO J* 13: 1189–1196.
- Sanlioglu S, Williams CM, Samavati L, Butler NS, Wang G, McCray PB *et al.* (2001). Lipopolysaccharide induces Rac1-dependent reactive oxygen species formation and coordinates tumor necrosis factor-alpha secretion through IKK regulation of NF-kappa B. *J Biol Chem* 276: 30188–30198.
- Shiraishi A, Miyabe S, Nakano T, Umakoshi Y, Ito M, Mihara M (2009). The combination therapy with alfacalcidol and risedronate improves the mechanical property in lumbar spine by affecting the material properties in an ovariectomized rat model of osteoporosis. *BMC Musculoskelet Disord* 10: 66.
- Smietana MJ, Arruda EM, Faulkner JA, Brooks SV, Larkin LM (2010). Reactive oxygen species on bone mineral density and mechanics in Cu,Zn superoxide dismutase (Sod1) knockout mice. *Biochem Biophys Res Commun* 403: 149–153.
- Sun Y, Shuang F, Chen DM, Zhou RB (2012). Treatment of hydrogen molecule abates oxidative stress and alleviates bone loss induced by modeled microgravity in rats. *Osteoporos Int* (in press).
- Talaulikar VS, Chambers T, Manyonda I (2012). Exploiting the antioxidant potential of a common vitamin: could vitamin C prevent postmenopausal osteoporosis? *J Obstet Gynaecol Res* 38: 253–257.
- Wimalawansa SJ, De Marco G, Gangula P, Yallampalli C (1996). Nitric oxide donor alleviates ovariectomy-induced bone loss. *Bone* 18: 301–304.
- Wolf RL, Cauley JA, Pettinger M, Jackson R, Lacroix A, Leboff MS *et al.* (2005). Lack of a relation between vitamin and mineral antioxidants and bone mineral density: results from the Women's Health Initiative. *Am J Clin Nutr* 82: 581–588.
- Zhang YB, Zhong ZM, Hou G, Jiang H, Chen JT (2011). Involvement of oxidative stress in age-related bone loss. *J Surgical Res* 169: e1–e6.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 Sequences of oligonucleotides used as primers.